REGULATION OF GASTRIC ACID SECRETION *in situ* BY AN ENDOGENOUS ACITVATOR PROTEIN: STUDIES WITH ACTIVATOR SPECIFIC ANTIBODY

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Summary

Monospecific polyclonal antibody was raised against a homogenous preparation of endogenous activator protein (HAF) for the gastric H+,K+-ATPase system. Antibody was used to assess the regulatory role of the HAF in gastric acid secretion by isolated rabbit glands in situ. Immunohistochemical studies revealed a redistribution of the HAF towards discrete intracellular zones following stimulation of the glands with histamine. The antibody, when inserted into the stimulated gastric glands by digitonin permeabilization, could effectively block the acid forming ability of the cells. The data offers, for the first time, some concrete in situ evidence for the role of the HAF as an intracellular regulator of gastric H+ transport.

Introduction

Gastric H+,K+-ATPase located at the apical plasmalemrna and the intracellular tubulo-vesicular membranes of the parietal (acid secreting) cells has been identified as the enzymatic mechanism for the active transport of hydrochloric acid from stomach (1, 2). Following stimulation of the parietal cells with secretagogues, the intracellular tubulovesicles fuse with the apical plasmalemrna providing both the extra surface area as well as the large number of proton pumps needed to meet the secretory demand of the tissue (1).

The mechanisms involved in the intracellular control of gastric acid secretion have been a subject of extensive investigation in recent years. Besides the well-known second messengers like the inositol phosphatides (3), cyclic AMP (4) and Ca+2 (5), an endogenous activator protein (HAF) capable of stimulating the gastric H+, K+,.A1Pase activity in vitro has been strongly implicated *to* be involved in the cytosolic regulation of gastric H+ transport (6). The HAF protein has recently been characterized following its purification to homogeneity (7, 8). Studies conducted with the pure HAF and highly purified preparations of the apical and the tubulovesicular membranes revealed that unlike the tubulovesicles, the apical plasmalemmal H+, K+-ATPase activity is solely

dependent on the HAF for activity (9). The HAF dependent H+, K+-ATPase activity has been demonstrated to show some unique behavior towards Ca+2 (8, 9). Thus Ca+2, at concentrations up

to 1m, is quite innocuous to the RAF-dependent H+, K+-ATPase activity while at higher (2-4 µm) concentrations, Ca+2 obliterates the HAP-dependent activity. It was suggested (89) that Ca+2 may be acting as a physiological switch for the gastric H+-transport process. The evidences (8) are also consistent with the idea that the RAF and Ca+2 are the two terminal members of an intracellular signal transducing cascade system involved in the stimulus-secretion coupling of gastric H+ transport .

In the present report we used the HAP-specific polyclonal antibody as a tool to gather more direct evidence for the role of the HAF as an intracellular regulator of gastric H+ transport. The data reveal that the antibody specifically blocks the secretagogue-stimulated component of gastric acid secretion by isolated rabbit gastric glands in situ. Immunofluorescence studies on the localization of the HAF in isolated gastric glands reveal that during secretagogue-stimulation the HAF molecules appear to be mobilized towards a specific area, presumably the secretory cannelicular region of the parietal cells. The data offer the first in situ evidence for the role of the HAF as an intracellular regulator of gastric H+ transport.

Materials and Methods

Preparation of Purified Gastric Membranes: Gradient purified pig gastric microsomes highly enriched in H+,K+-ATPase and pNPPase activities were isolated from fresh pig fundic mucosa following our previously published procedure (10). The microsomes were subsequently subfractionated into the apical plasmalemmal and the tubulovesicular membranes by treatment with low concentration sodium dodecyl sulfate followed by sucrose density gradient centrifugation (9).

Purification of the Activator Protein (HAF):The activator protein was isolated from pig fundic cells following our recent procedure (7,8).

Isolation of Rabbit Gastric Glands: gastric glands are prepared (11) from male New Zealand white rabbits following the Berglindh procedure (2). Briefly, the stomach of the Nembutal anesthetized animal is perfused under pressure with phosphate buffered saline. the fundic mucosa is scraped off, minced and digested with lmg/ml collagenase (Sigma, Cat# C-0130) with stirring at 30° C for 45-60 min under 95% 02-5% C02 (without bubbling). The collagenase digestion medium consists of.(in mM) 130 NaCl, 12 NaHC03, 3 Na2HP04, 2 K1HP04, 2 MgS04 and 1Ca+2 (pH adjusted 7.4). Following filtration of the digest through wide mesh gauze, the glands are harvested by centrifugation at 200 x g for 5 min. The glands are then resuspended in normal incubation medium and allowed to settle for 10 min. This is followed by two additional resettlings in normal medium. The normal incubation medium consists of (in mM) 132.4 NaCl, 5.4 KCI, 5 Na2HP04, 1 Na2H2P04, 1.2 MgS04, 1 CaCl2, 1 Pyruvate and 2mg/ml BSA (pH 7.2). After the settling, the glands are suspended in incubation medium at a final concentration of 10 mg wet wt/ml and kept at room temperature until use.

Preparation of Polyclonal Antibody: Monospecific polyclonal antibody against a homogenous preparation of pig HAF was raised in young female rabbits. Pure HAF in complete Freund's adjuvant was injected into two female rabbits (200 µg HAF protein/animal) at 30-40 different sites. A second injection was given in the same way after 3 weeks. Nine weeks later, 100 µg of the antigen was given as a booster. Antibody production was monitored by Western blot.

Measurement of Acid Formation in Digitonin Permeabilized Gastric Glands: Isolated gastric glands from rabbits were used to measure acid secretion in situ (13) following permeabilization with digitonin in the absence and presence of the anti-RAF antibody. The ability of the glands to form acid was measured as the ratio of [14C] aminopyrine accumulation between the extraglandular and intraglandular water following the procedure of Berglindh (12). The accumulation of [14C] aminopyrine by the digitonin premeabilized glands was measured in the presence of ATP and high (150mM) K+ in the medium following the procedure of Hersey and Steiner (13). The high-K+ medium used for the incubation contained (in mM) 100 KCl, 1.2 MgCl2, 40 mannitol, 50 HEPES,

0.5 DTT, 1 NaH2P04, 1pyruvate and 10 succinate; the pH being adjusted to 7.4 with NaOH yielding a Na+ concentration of -25 mM.

Permeabilizatjon of Gastric Glands to anti-HAF Antibody: Aliquots of the glands were first incubated for 20 min at 37°C in the presence of stimulants, histamine (10-4M) plus isobutyl methyl xanthine (10-5M), or the secretory antagonist cimetidine (2x104 M). Following this treatment the glands were exposed to digitonin (20µg/ml) in high-K+ medium for 20 min at 21°C. The digitonin treated glands were washed once and then resuspended in fresh high-K+ medium containing either the desired dilution of anti-HAF antibody or an equivalent amount of BSA. After 20 min at 21°C, aliquots of the glands (20 mg wet wt) were incubated for 15 more min at 21cwith 1 mM ATP and 0.1 µCi/ml of [14C] amino-pyrine in the high-K+ medium. At the end of incubation the glands were pelleted by centrifugation and counted by liquid scintillation.

Assay of H, K-ATPase Activity: The ATPase activity was assayed as previously described (8). For activator activation of the H+,K+-ATPase, the membranes were first pre-incubated for 10 min at 37°C without (control) and with the desired concentration of activator protein. Aliquots of the pre-treated membranes were used as a source of the enzyme.

Western Blot Analysis: SDS-PAGE was performed according to Laemmli (14). Proteins were electrophoretically transferred to nitrocellulose using 48 mM TRIS, 39 mM glycine (pH 8.8) containing 20% methanol and 0.1% SDS. Western blots were probed with goat anti-rabbit IgG linked with peroxidase.

Imrnunohistochemistry: Isolated gastric glands were either stimulated with histamine (104M) plus isobutyl methyl xanthine (10-5M) or inhibited with cimetidine (2xl0-4M) at 37°C as described earlier. All further procedures were conducted at 21"C. The glands were washed once with PBS and fixed for 20 min in 4% formaldehyde following the procedure of Hanzel *et al* (15). The fixed glands were permeabilized with 0.5% Triton X-100 in PBS for 20 min with agitation. Following three washes in PBS, the permeabilized glands were settled on polylysine coated cover slips and incubated with 1% BSA and 0.05% Tween 20 in PBS for 30 min. The glands were then exposed to 1:100 dilution of the anti-HAP antibody for 60 min. Following three 15 second washes with
0.05% Tween 20 in PBS, the anti-RAF antibody was localized by incubating for 60 min in fluorescein isothiocyanate conjugated goat anti-rabbit lgG (1:250 dilution in 1% BSA. 0.05% Tween 20 in PBS).

Results and Discussion:

A. Specificity of the anti-HAF Antibody: The polyclonal antibody was raised in rabbits against a homogenous preparation of pig HAF (Fig. 1). The antibody preparation was found to be specific for the HAF based on the following criteria. It specifically inhibits the HAF stimulated component

of the H+, K+-ATPase activity without having any effect on the basal (Mg+2-dependent) or the HAF-independent component of the H+, K+-ATPase activity (Fig. 2) The observed inhibition was not due to any nonspecific interaction of the antibody with the H+, K+-ATPase molecule *per se* as revealed by the lack of ability of the a and p subunits of the H+, K+-ATPase to interact with the antibody on Western blot (Fig. 3). The antibody primarily recognizes two closely apposed bands near the 40 k Da region. The low molecular weight peptides are most likely the products of proteolytic degradation of the membrane-associated HAF molecules and appeared as artifacts in this particular preparation. The appearance of double bands in the 94 k Da catalytic subunit region will be consistent with such an idea since a single band is normally observed in our preparations (9). It may be noted in this connection that our cleanest preparation of tubulovesicular membranes show only α and β subunits of the H, K-ATPase on SDS-PAGE (9). Hence, the observed presence of the antibody-recognizable low molecular weight peptides in the tubulovesicular fraction is most likely due to some cross-contamination from the apical plasmalemmal fraction. In light of our current knowledge and as discussed. below, the observed preferential association of the HAF molecules with the apical or the secretory cannelicular membranes is not surprising.

The apical membranes are believed to be the sole sites for translocation of the protons following secretagogue-stimulation. Because the apical plasmalemmal H+. K+-A1Pase system is almost solely dependent on the HAF for activity it is anticipated that the apical plasmamembranes would have preferential affinity for the HAF molecules. Since the lipid composition (9) of the apical and the tubulovesicular membranes are both qualitatively and quantitatively different, it is possible that the HAF binding ability may be attributed, at least in pan. to the phospholipid makeup of the apical membranes. Also, the low or possible lack of affinity of the tubulovesicles for HAF binding may have important physiological consequences. Thus, a portion of the RAF-dependent H+, K+-ATPase molecule observed. to be present (9) in the tubulovesicular fraction will remain unoperative if the tubulovesicles are unable to sequester the HAF molecules. Our data (see below) on immunohistochemical localization of the HAF reveal mobilization of the HAF molecules towards the secretory cannelicular region following secretagogue-stimulation of the cells (Fig. 3).

B. Immunohistochemical Localization of the Intracellular HAF: The HAF-specific antibody, described above, was subsequently used for intracellular localization of the HAF under both resting and stimulated conditions. As evident from Fig. 3, there is a significant rearrangement in the distribution of the HAF molecules following stimulation of the parietal cells by histamine and isobutyl methyl xanthine. The fluorescent HAF molecules are mobilized and are concentrated in discrete areas which appear as brightly spotted zones within the cytoplasm. Such bright spotted areas are most likely the cannelicular regions of the parietal cells where the H-pump molecules are relocated following stimulus-induced tubulovesicular fusion.

C. Antibody Inhibition of Gastric Acid secretion by Isolated Rabbit Gastric Glands in situ: The RAF-specific antibody was subsequently tested for its effects on acid secretion by the freshly isolated permeabilized rabbit gastric gland preparation using the standard technique of [14C] aminopyrine accumulation ratio (12,13). The results, depicted in figure 5, show that the HAF- specific antibody inhibits the aminopyrine accumulation ratio in a dose-dependent manner. The secretagogue-stimulated acid secretion is nearly abolished in the glands exposed to 1:50 dilution of the antibody. It may be pointed out that a small but appreciable portion of the aminopyrine accumulation within the non-stimulated as well as the stimulated glands resists antibody inhibition. This antibody-resistant component is most likely due to an RAF-independent aminopyrine accumulation within the cytosolic tubulovesicles. Recent demonstration of the exclusive presence of an HAP-independent H+. K+-ATPase in a highly purified tubulovesicular fraction (9) will be consistent with such a conclusion. Antibody inhibition of acid secretion by the isolated glands provides, for the first time, a direct evidence for the role of the HAF as an intracellular regulator of gastric H+ transport.

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Figure Legends

Figure 1: Western blot analysis of homogenous HAF preparation from pig fundic mucosa. The figures on the left (a) are for coomassie blue stain and, on the right (b), for antibody' stain. a) The lanes from left to right are 4µg, 8µg and 12µg of the HAF in the first and second and third lanes respectively; the fourth lane contains 50µg of dog gastric mucosa homogenate, and the fifth and sixth lanes represent high and low molecular weight standards respectively. b) Western blot of duplicate gels run under identical conditions as in (a) above, without the standards. The details for anti-body staining are given in method s. As expected, the concentration of HAF in the homogenate is very low. However, high specificity of the antibody in recognizing the RAF is clearly evident.

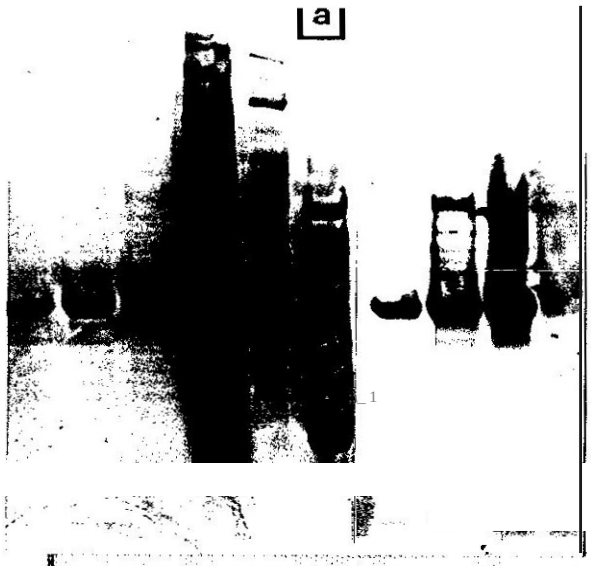
Figure 2: Effects of different dilutions of the anti-HAF antibody on the RAF-dependent H+,K+- A1Pase activity associated with the purified secretory (apical) membranes of the parietal cells. Aliquots of a pure preparation of pig HAF containing 50µg of activator protein were incubated at 0-4° C for 1 hour with different dilutions of the antibody. Following a brief centrifugation (5000 g for 5 min) in cold, the supernatant was assayed for activator activity using 5µg of purified apical plasma membranes at a membrane to activator ratio of 1:2 on a protein basis. The details of the ATPase assay are given in methods.

Figure 3: Western blot analysis of various membrane sub-fractions showing antibody recognition of the membrane associated HAF. The figure on the left shows the amide black stain for protein following transfer to nitrocellulose paper, and on the right the Western blot. The abbreviations are MW, molecular weight standards; MC, microsomes; AP, apical plasma membrane fraction; and TV, tubulovesicular membrane fraction. Please note that the antibody does not recognize the 94 k Da H+,K+-ATPase catalytic subunit region. The antibody primarily recognizes two closely apposed bands near the 40 k Da region and a few other minor peptides of lower molecular mass.

Figure 4: Immunohistochemical localization of the intracellular HAF in rabbit cells. Bright field view (with phase contrast optics) of the unstimulated gastric glands (a), and stimulated gastric glands (c); (b) and (d) are the corresponding fluorescent micrographs stained with anti-HAF antibody and FITC conjugated goat anti--rabbit IgG respectively. The details are provided in the methods. Two sets of controls were run-- one being unexposed to the primary antibody and the other to the second antibody; all other steps being the same as the experimental groups. The control cells did not show any characteristic FITC fluorescence (data not shown). Magnification: x40.

Figure 5a: Antibody inhibition of acid secretion by isolated gastric glands in situ. Aliquots of the freshly isolated rabbit gastric glands were separately incubated with cimetidine to bring to a resting state or stimulated with histamine plus isobutyl methyl xanthine (IBMX) as detailed in methods. The resting and stimulated glands were then treated with digitonin (20µg/ ml) for 20 min. Following a brief wash (1000 g, 5 min), the permeabilized glands were suspended in "high K+" medium, without and with the antibody. The glands were then measured for (14C] aminopyrine accumulation in presence of ATP as detailed in methods. Data are mean ± SEM.

Figure 5b: Dose response of anti-HAF antibody on [14C] aminopyrine uptake. Please note that the antibody specifically abolishes the hormone stimulated component of aminopyrine uptake without having any appreciable effect on the resting aminopyrine ratio except at high concentrations (1:50) of the antibody. The details of the experiments were identical to those given in 5a except that the antibody was used at several different dilutions.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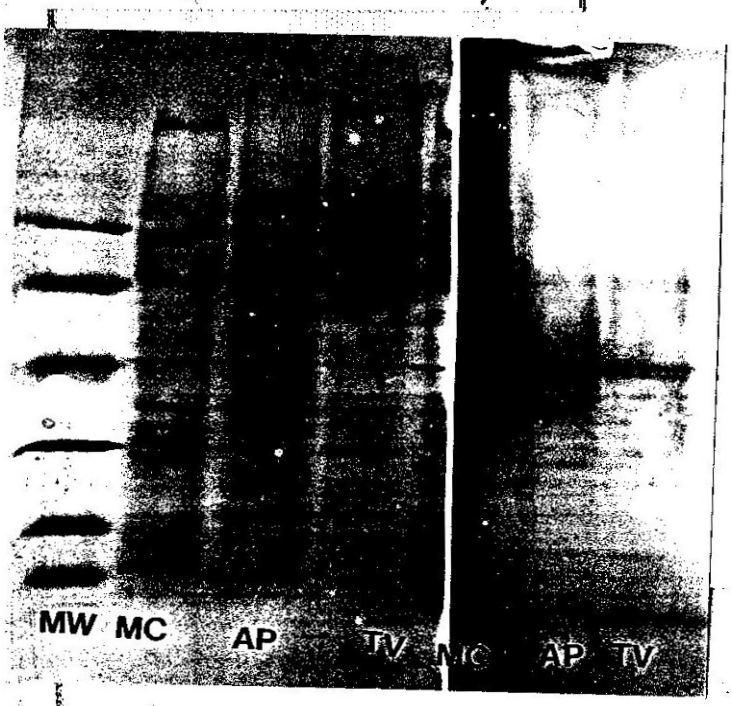
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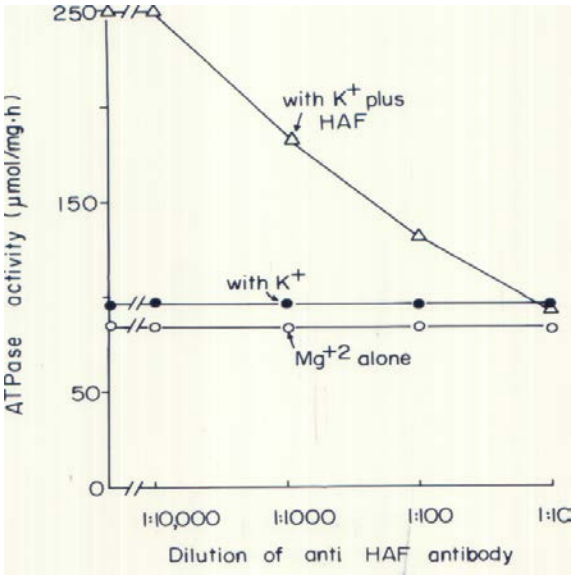
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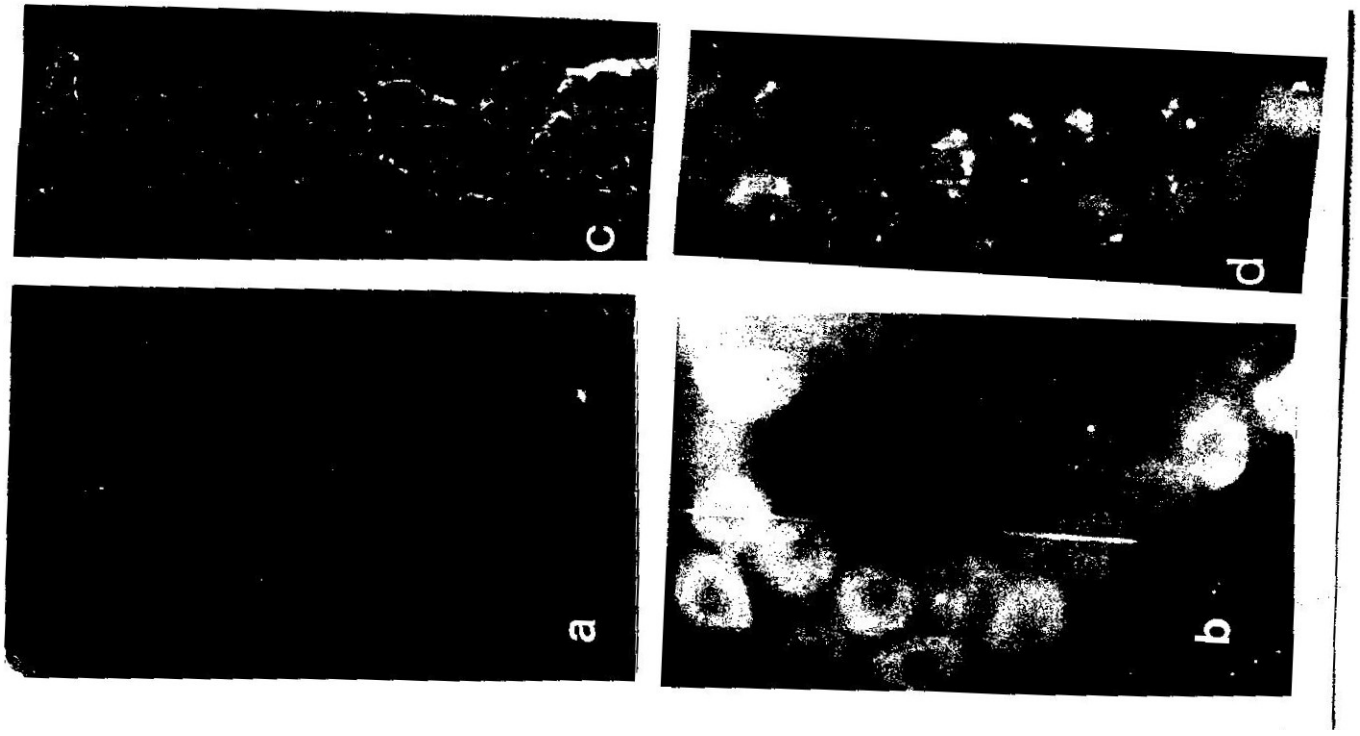
Dilution of anti HAF antibody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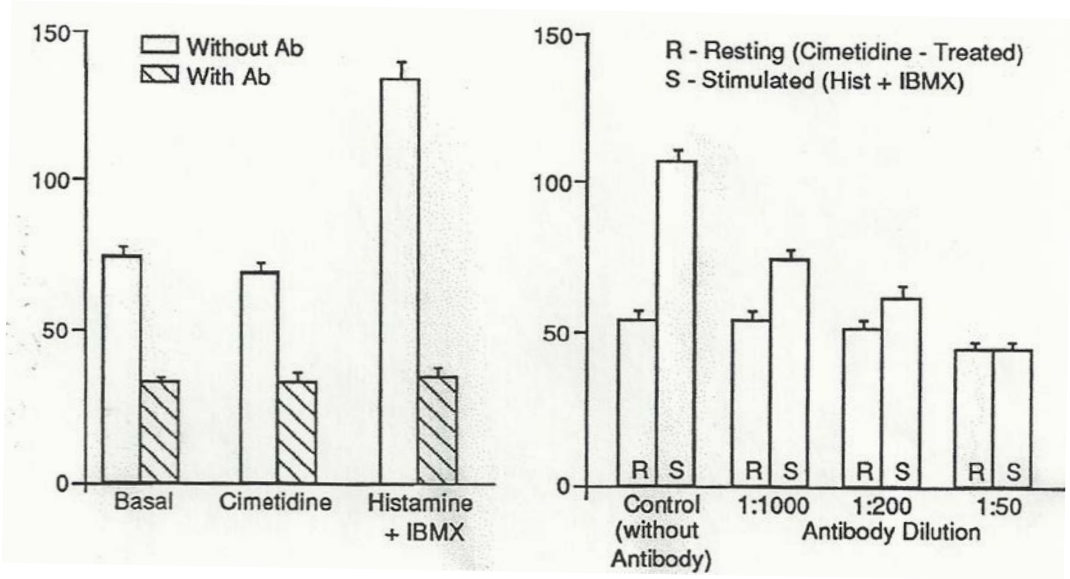
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